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Biology and genetic engineering of fruit maturation for enhanced quality and shelf-life

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Commercial regulation of ripening is currently achieved through early harvest, by controlling the postharvest storage atmosphere and genetic selection for slow or late ripening varieties. Although these approaches are often effective, they are not universally applicable and often result in acceptable, but poor quality, products. With increased understanding of the molecular biology underlying ripening and the advent of genetic engineering technologies, researchers have pursued new strategies to address problems in fruit shelf-life and quality. These have been guided by recent insights into mechanisms by which ethylene and a complex network of transcription factors regulate ripening, and by an increased appreciation of factors that contribute to shelf-life, such as the fruit cuticle.

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Fruits are important contributors to human diets and health, providing essential nutrients, antioxidants, carbohydrates, and fiber. The ripening process has evolved to make fruit palatable to organisms that consume them and disperse their seeds. In doing so, ripening activates pathways that generally influence the levels of pigments (typically carotenoids and flavonoids), sugars, acids, and aroma volatiles, to make the organ more appealing, while simultaneously promoting tissue softening and degradation to permit easier seed release [1,2]. Indeed the volatiles produced by ripening fruit are also derived from, and represent signals for, the presence of essential nutrients for animals which may consume them [3]. Increased susceptibility to postharvest microbial infection helps insure that the seed are released via fruit rot, if not consumption. A major challenge to breeders and producers of fruit species continues to be how to capture and deliver to market the desirable flavor, color, and texture attributes of ripe fruit while inhibiting or delaying ripening sufficiently to counter the negative consequences of over-ripening: oversoftening and decay.

Ethylene and fruit ripening

Ripening is regulated by both internal and external stimuli, including temperature, light, plant nutrient status, water availability, and hormones. Many fruits, in particular the so-called climacteric fruits, require ethylene for ripening, resulting in the targeting of this hormone as a means of ripening control. Although nonclimacteric fruits typically produce little ethylene during ripening, many have still been shown to be affected by exogenous ethylene during ripening, making ethylene control a target for shelf-life manipulation even in species whose fruit do not require ethylene to ripen ([1,2] and references therein). Because of the importance of ethylene for ripening, the genes responsible for its synthesis, ACC synthase (ACS) and ACC oxidase (ACO), were early targets of study and manipulation and repression of using antisense strategies delayed ripening in either tomato or other species (reviewed in [2]). Commercialization of these technologies has been limited by the cost of regulatory compliance, real or perceived consumer concerns, and the efficiency of available postharvest ethylene control systems.

Ethylene response genes: potential targets for ethylene response and ripening control

Ethylene signal transduction genes have been well characterized in *Arabidopsis* (for recent reviews see [4–6]). More recent efforts in understanding the ethylene response during fruit ripening have focused on the characterization of tomato homologs. All the components of the *Arabidopsis* ethylene signal transduction analyzed thus far are conserved in tomato (reviewed in [2]). However, the family size and expression profiles of some of these genes differ between *Arabidopsis* and tomato. For example, there are six ethylene receptors in tomato, compared to five in *Arabidopsis*, but all have similar binding affinities for ethylene. Further, expression studies indicate distinct profiles for *Arabidopsis* and tomato ethylene receptor genes. In *Arabidopsis* the *CONSTITUTIVE TRIPLE*

RESPONSE1 (*CTR1*) MAP kinase kinase kinase is represented by a single locus, whereas in tomato a family of three genes exists, all of which can complement the *Arabidopsis* *ctr-1* loss-of-function mutation. The additional components of ethylene signal transduction in tomato point to more opportunities for fine-tuning regulation of the hormone response than in *Arabidopsis*. Recently, an exploration of the protein–protein interactions of tomato ethylene receptors and the downstream kinases revealed that a single ethylene receptor, *NEVER RIPE* (*NR*), is capable of interacting with multiple LeCTR proteins [7•], supporting the hypothesis that ethylene receptors transmit the signal to downstream CTRs. However, it is still unclear whether members of the tomato CTR family are functionally redundant or elicit unique ethylene responses.

Recently, novel ethylene signaling components were discovered by the simultaneous cloning of the tomato *GREEN RIPE* (*GR*) and *Arabidopsis* REVERSION TO ETHYLENE SENSITIVITY1 (*RTE1*) loci. The gene responsible for the dominant ripening mutation in tomato was positionally cloned [8], whereas *RTE1* was discovered through a second-site suppressor screen of an *Arabidopsis ETHYLENE RECEPTOR1* (*ETR1*) mutant [9]. There are at least two other GR-like genes in the tomato genome (*GRL1* and *GRL2*), but only one other family member of RTE1, designated RTH, is present in *Arabidopsis*. Characterization of the *GR* mutation revealed a deletion of noncoding 5′-upstream sequence that results in fruit-specific ectopic expression of this gene during early development and throughout ripening. This overexpression causes a severe reduction in ethylene sensitivity, which in turn results in drastic ripening inhibition even though normal or elevated ethylene levels are produced [10]. Constitutive overexpression of GR in transgenic plants caused inhibition of ripening but surprisingly little, if any, altered ethylene sensitivity was observed for other aspects of plant development. This suggests GR might interact with components of the fruit-specific ethylene response. Furthermore, the fact that the ethylene response inhibition results from ectopic expression, rather than targeted gene repression, suggests that the tomato gene might be functional in other species merely through overexpression.

The gene underlying the original ethylene-insensitive *Etr1* ethylene receptor mutation was isolated from *Arabidopsis* and shown to encode a two-component receptor kinase that binds ethylene (reviewed in [1,2]). The mutation in this and many other dominant ethylene-insensitive receptor alleles impaired the ability of the receptor protein to bind ethylene. Thus, another strategy for blocking ethylene perception in fruit could be at the receptor itself. Indeed, overexpression of the mutant ETR1 protein resulted in delayed fruit ripening in tomato and inhibition of petal senescence in petunia. The tomato *Never-ripe* (*Nr*) mutation was also shown to encode an ERS-like ethylene receptor that is also impaired in the ability to bind ethylene. Transcript levels of *LeETR1*, *LeETR2*, and *LeETR5* change little upon treatment of ethylene in fruit, whereas *NR*, *LeETR4*, and *LeETR6* increase during ripening, suggesting that they may be more important in fruit [11]. Reduction of either of two ethylene receptors, *LeETR4* or *LeETR6*, causes an early ripening phenotype in tomato, in agreement with models where ethylene receptors act through kinase-mediated inhibition of the negative regulator CTR1 [11]. Further, the receptor proteins are targets of the 26S proteosome-dependent pathway, and are degraded in the presence of ethylene. In addition, exposure of immature fruits to ethylene caused a reduction in the amount of ethylene receptor protein and earlier ripening, supporting the hypothesis that receptor levels regulate the timing of the onset of fruit ripening by measuring cumulative ethylene exposure [11]. Recently a tetratricopeptide repeat protein (SlTPR1) was isolated from tomato and shown to interact in yeast two hybrid assays with NR and LeETR1 proteins [12]. This class of protein is involved in the ubiquitination process which leads to proteosome-mediated protein degradation. Ethylene and auxin responses were modulated in plants overexpressing SlTPR1; however, fruit ripening, as monitored by color and textural changes, was not affected. It is proposed that a similar and specific protein–protein interaction occurs for LeETR4 and LeETR6 to regulate receptor levels during tomato fruit ripening. In summary, the receptors, and the proteins with which they interact, provide additional targets for the manipulation of ethylene responses, including ripening. The broad array of responses regulated by ethylene suggests that for such genes to be effective in ripening control they would need to be modified in a fruit-specific manner.

Transcriptional control of fruit ripening and conserved ripening regulators

Although considerable effort has focused on ethylene synthesis and response, only recently have inroads been made into understanding ripening control before ethylene: a regulatory system that is possibly conserved between climacteric and nonclimacteric species. Recently, the mutated genes underlying the ripening mutants *RIPENING INHIBITOR* (*RIN*) and *COLOURLESS NON RIPENING* (*CNR*) were cloned, providing key insights into the regulation of the ripening process upstream of ethylene. The *rin* and *Cnr* mutations are recessive and dominant mutations, respectively, which block the ripening process and result in fruit that are unable to respond to ripening-associated ethylene [13,14]. The *rin* mutation is especially interesting as it has been bred into many commercial tomato varieties to slow ripening and extend shelf-life. Both *rin* and *Cnr* encode transcription factors, providing insights into dedicated fruit-specific control of ripening. *rin* encodes a

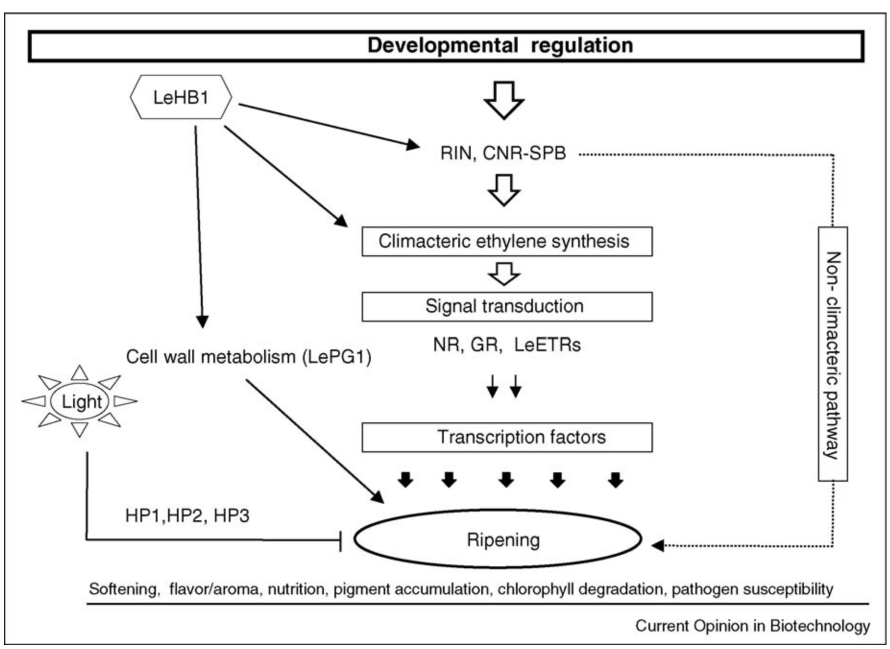
MADS-box protein of the SEPALLATA clade [15], while *Cnr* is an epigenetic mutation that alters the promoter methylation of a SQUAMOSA promoter binding (SBP) protein. Both these transcription factors are required for ripening-associated increases in respiration and ethylene; characteristics of tomato and other climacteric fruit species. Furthermore, fruits from both mutants remain responsive to ethylene (even though they do not ripen), in that ethylene-responsive genes are induced by exogenous ethylene. The fact that they do not ripen in response to the hormone indicates that both genes operate upstream of crucial ripening activities, including ethylene biosynthesis and necessary activities that are apparently ethylene independent (Figure 1). This observed ethylene-independent aspect of ripening physiology suggests that both RIN-MADS and CNR-SBP proteins could be conserved ripening regulators of fruit in both the climacteric and nonclimacteric categories. Strawberry is the most widely studied species of the nonclimacteric classification. A ripening-related strawberry homolog of the tomato *RIN* gene has been isolated, which is consistent with the hypothesis that such regulators might be conserved between both climacteric and nonclimacteric fruits [13]. *RIN* and *CNR* may therefore represent good candidates for ripening control in a broader array of *species* than is currently possible via ethylene control alone.

Another tomato transcription factor, *LeHB-1*, has also recently been characterized and shown to play a central role in fruit ripening [16••]. *LeHB-1* encodes a putative HD-Zip homeobox protein that binds to the promoter of ACC oxidase (*LeACO1*), which encodes a key enzyme in the ethylene biosynthetic pathway. Antisense inhibition of LeACO1 expression was reported to result in reduced ethylene synthesis and in this recent study, transient silencing of *LeHB-1* resulted in a significant delay of ripening and a reduction of *LeACO1* transcript abundance [16••]. Together these observations support the idea that LeHB-1 positively regulates LeACO1 and that silencing of LeHB-1 represses LeACO1, consequently leading to a delay in ripening. Putative LeHB-1-binding sites are also present in a number of other ripening-related genes including *LeACO2*, *LePG1*, and *LeMADS-RIN* and so it is possible that LeHB-1 might also regulate these ripening-related genes directly (Figure 1).

Genetic manipulation of ripening regulatory genes

To date, attempts to manipulate the expression of target genes during fruit ripening have produced varying results. As mentioned, transcriptional control has been utilized with respect to breeding for heterozygosity at the *rin* mutation (*Rin/rin*) in commercial lines to delay ripening and extend shelf-life; however, this modification leads to the inhibition of flavor and nutritional compound accumulation along with undesirable textural traits in some backgrounds due to incomplete ripening. In addition, constitutive expression or silencing of other

Figure 1



Model for the molecular regulation in tomato ripening. This model was updated from [2] with LeHB-1 elucidated by [16••]. The promoters of *LeACO1*, *LeACO2*, *LePG1*, and *LeMADS-RIN* contain a putative LeHB-1-binding site suggesting the LeHB-1 transcription factor may universally control fruit ripening as well as floral organogenesis and carpel development.

target genes can cause unwanted pleiotropic effects on other aspects of plant development, or even lead to lethality as is probably the case for the *Cnr* and *LeHB-1* loci, as stable transgenic plants harboring either of these genes have been difficult to produce. As a consequence of unwanted pleiotropic effects, targeted analysis of the transgene effect in the fruit is often difficult, as exemplified by the light signal transduction and carotenoid regulatory gene *HIGH-PIGMENT2*, where pleiotropic effects of transgene expression in nonfruit tissues were noted (low yield and brittleness), although this can be overcome through the use of a fruit-specific promoter [17]. More accurate phenotypic determination of fruit function and utility for the assessment of other production or quality influences will be achieved through the use of appropriate tissue-specific and development-specific promoters. Indeed, the most useful transgenic or natural alleles for ripening control will be those that deliver fruit specificity and result in levels of expression sufficient for extended shelf-life while ripening sufficiently for desirable quality. While several fruit-specific promoters are known, the commercial use of most is subject to intellectual property restrictions and even they represent a narrow range of expression options (essentially either throughout fruit development or induced at the onset of ripening). Isolation and utilization of promoters with more desirable temporal or spatial expression profiles could provide the means to regulate fruit ripening by manipulating the expression of transcription factors such as *rin*, *Cnr*, and *LeHB-1*, absent unintended effects. Further, more detailed characterization of the molecular make up of ripening regulatory networks [18] should provide additional fruit-specific loci which can be altered genetically to avoid undesirable secondary effects, without the need of fruit-specific promoters. Some examples with particular reference to altering cell wall metabolism and texture are explored in subsequent sections.

Cell walls and cuticles as key factors for fruit shelf-life

The cell wall has a profoundly important influence on fruit texture and cell wall components and the underlying genes have been frequent targets for genetic engineering, mostly in tomato, with the goal of extending shelf-life [19,20]. Although knowledge of the mechanism of fruit softening has grown in recent years, it has proven especially difficult to establish the relationship between specific aspects of cell wall metabolism or architecture and their relationship to changes in tissue firmness [19,21]. Similarly, it has also been difficult to establish the fine regulation of genetic elements that produce a range of effects depending on the genetic background in which they are introduced [22]. The regulation of texture and shelf-life is clearly far more complex than was previously envisaged and so new approaches are needed, including the inclusion of observations in species beyond the traditional tomato experimental model [23]; a better understanding of the relationship between changes in the textural properties of specific fruit tissues, intact fruit 'firmness' and shelf-life; and more comprehensive models of the biochemical and physiological elements that contribute to fruit 'firmness'.

Ripening-related disassembly of the cell wall polysaccharide matrix is generally the only factor that is mentioned when describing the structural basis of fruit softening and the associated loss of shelf-life and fruit quality. This is largely a reflection of the relative ease with which some of the dramatic changes in wall polymer structure and composition, and the activities of the associated enzymes, can be measured. However, recent studies have started to examine the potential involvement and relative importance of other structures and physiological processes. For example, it has been proposed that differences in tomato fruit cuticle structure and composition may be associated with the substantial variation in tomato fruit shelf-life that has been reported in different tomato genotypes [24•]. The cuticle has a number of biological functions that could have an important impact on fruit quality and shelf-life, including the ability to maintain fruit skin integrity [25], restrict cuticular transpiration [26•], and limit microbial infection. Other reports have also highlighted ripening-related processes that likely contribute to fruit firmness, such as turgor pressure [24•,27•] and the possibly associated developmental changes in apoplastic solute accumulation [28]. Integration of all these features into a more holistic model of ripening-related textural changes should provide a host of new targets for gene manipulation, with the ultimate goal of allowing more complete fruit ripening on the plant, thereby promoting nutrient and flavor accumulation while maintaining fruit firmness and shelf-life.

When a fruit is more than a fruit?

Fresh fruits and their processed derivates represent important sources of carotenoids, flavonoids, and anthocyanins and the possibility of improving content using bioengineering represent an opportunity to improve access to healthy foods. The inherent resiliency of plant metabolism to maintain homeostasis has impaired efforts to facilitate changes in some of these pathways, as has been especially well documented for carotenoids [29]. An alternate approach is to focus on regulatory rather than biosynthetic genes. Accumulation of carotenoids in high-pigment fruit mutants has been reported to result from an increase in the plastid number, larger plastid compartment size, increased plastid division, and a greater capacity for pigment storage [30,31]. These changes in pigmentation, plastid type, and metabolism were associated with the elevation of transcripts from key carotenoid genes such as phytoene synthetase-1 (*Psy-1*) and are not directly connected with the ripening process [32], thus allowing modifications in carotenoid content without affecting other aspects of fruit ripening or broader plant development. Moreover, this particular modification has been reported to be stable in field tests for transgene stability and yield performance [33].

Recently, a MYB transcription factor from snapdragon was expressed using the E8 promoter specifically in tomato fruit, causing elevated levels of anthocyanins: compounds that have been shown to increase the longevity of cancer-susceptible mice [34••]. A homolog of this transcription factor (MYB10) was previously cloned and characterized from apple and was shown to positively regulate the anthocyanin pathway [35]. Further, an allelic rearrangement in the promoter of MYB10 causes a novel autoregulatory motif, which is sufficient to account for the increase in MYB10 levels and subsequent ectopic accumulation of anthocyanins throughout the plant [36]. This modification was found in all red apple species tested, but not in white apple varieties. This gain-of-function mutation in the anthocyanin regulatory pathway has significant implications for the development of novel varieties of plants and fruit with enhanced nutritional status and increased consumer appeal, by using either genetic modification or by more conventional breeding methods.

Examples of specific pathways that have been targeted through genetic engineering with either enhanced shelf-life or nutritional status, or genotypes of particular interest are shown in Table 1. However, impacts on health and nutrition can be mediated through less direct means than the alteration of nutrition associated biochemical pathways. These goals can be met by modifying overall appeal and quality so that fruit and fruit products become more competitive choices for consumers and net fruit consumption is increased. For example, seedless tomato fruit were developed by silencing a key enzyme of the flavonoid biosynthesis pathway [37].

New tools, new strategies, and enhancement of traditional approaches

Prior efforts toward fruit quality improvement have focused on single steps in biochemical pathways, but complex feedback regulation networks have complicated such strategies, as noted above. Targeting regulatory genes may provide a route to avoid these regulatory impediments though the difficult work of assessing the impact of altering specific pathway steps is required to understand the underlying regulatory networks [32]. The use of a transcription factor that regulates a complete biosynthesis pathway, as opposed to targeting a single enzyme, has proved useful to study and manipulate the accumulation of tannins in grape [38] or polyphenolic antioxidants in tomato [39]. New high-throughput functional genomics and systems approaches are also allowing more accurate predictions of regulatory processes [40]. Applying such methodologies to mapping of quantitative trait loci (QTL) allowed for the detection of epistatic interactions among genes [41•], the discovery of new genes related with metabolic pathways of interest, and even testing of the stability of QTL effects over multiple seasons and in different environments [42].

Recent reports revealed that the screening for genes whose expression varies between lines that are genetically very similar, and yet varying significantly in fruit quality, is a helpful tool for identifying potentially valuable genetic targets [43], and provides insights concerning the nature of complex genetic control processes that relate to fruit nutrient content [44], ripening, and softening [45]. These tools make it possible to identify potential candidate genes in model plants, like *Arabidopsis*, which could lead to targeting important genes in crop species [46]. In one interesting example, tomatoes were biofortified with calcium by the overexpression of an *Arabidopsis* calcium transporter (*CAX1*) [47]. However, while the calcium levels were increased, the bioavailability of the increased calcium was questioned. Similar experiments were carried out,

Table 1

Recent transgenic and mutant tomato and grape genotypes and their function/effects associated with nutrition or shelf-life

	Gene or mutant name/ID[a]	Biological process	Refs
Shelf-life			
β-Ketoacyl-CoA synthase	*LeCER6*/3760026	VLFC-cuticular waxes	[26•]
Cuticular water permeability	*Cwp1*/100136893	Cuticle microfissuring and dehydration	[25]
Delayed fruit deterioration	*dfd*	Extended shelf-life, reduced water loss	[24•]
Ethylene receptor	*LeETR4/543588*	Early-ripening fruit	[53]
Auxin response factor	*DR12*	Modification of fine pectin structure and tissue architecture	[21]
Nutrient content			
Chalcone synthase	*Chs*/778294, 778295	Flavonoid content reduction, parthenocarpic fruit	[37]
Phytoene synthase-1	*PSY-1*/543988	Changes to pigmentation, plastid type, and metabolism associated with Psy-1 overexpression	[32]
Isoflavone synthase	G*mIFS2/606705*	Presence of genistin in leaves	[54]
MYB transcription factor	*VvMYBPA1*/100232899	Specific regulation of proanthocyanidin biosynthesis	[38]
Flavonol-specific transcriptional activator	*AtMYB12*/819359	Flavonol accumulation in fruits	[39]

[a] Gene ID from www.ncbi.org.

where the intake of calcium from transgenic carrots over-expressing a *CAX1* gene were monitored in both mice and humans [48]. Calcium absorption in bones increased for both mice and humans eating CAX1-expressing carrots compared to controls, demonstrating alternative means of fortifying fruits and vegetables with bioavailable calcium.

Prospects for the future

It is important to note that recent studies have reported that genetically modified organisms are not inherently dangerous and can be considered safe to consumers when compared with conventional foods developed for target traits through naturally occurring genetic variation [49]. Of course, given that the safety of crops is a primary concern for both producers and consumers, it is necessary to define the risk and unintended effects in genetically modified plants [50], including the effects of a specific gene and the possible effects in a susceptible subgroup of consumers [51]. Although many of the target genes that result from emerging genomics approaches may not in themselves be candidates for direct manipulation, they may represent DNA sequences that can be exploited as markers in breeding programs to select for allelic variation, which can be tested and potentially used for the alteration of target traits. Selection of QTLs in particular can be made much more efficient through marked-assisted selection (MAS). Integrating MAS in traditional breeding practices is most likely the highest short-term impact of our rapidly increasing understanding of fruit molecular biology, as it is economically attractive and applicable for markets in both developed and developing countries [52]. The current proliferation of genetically modified cereal and fiber crops is likely to alleviate consumer concerns, reduce the current regulatory costs, and result in more direct biotechnology applications to fruit crops in coming years.

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